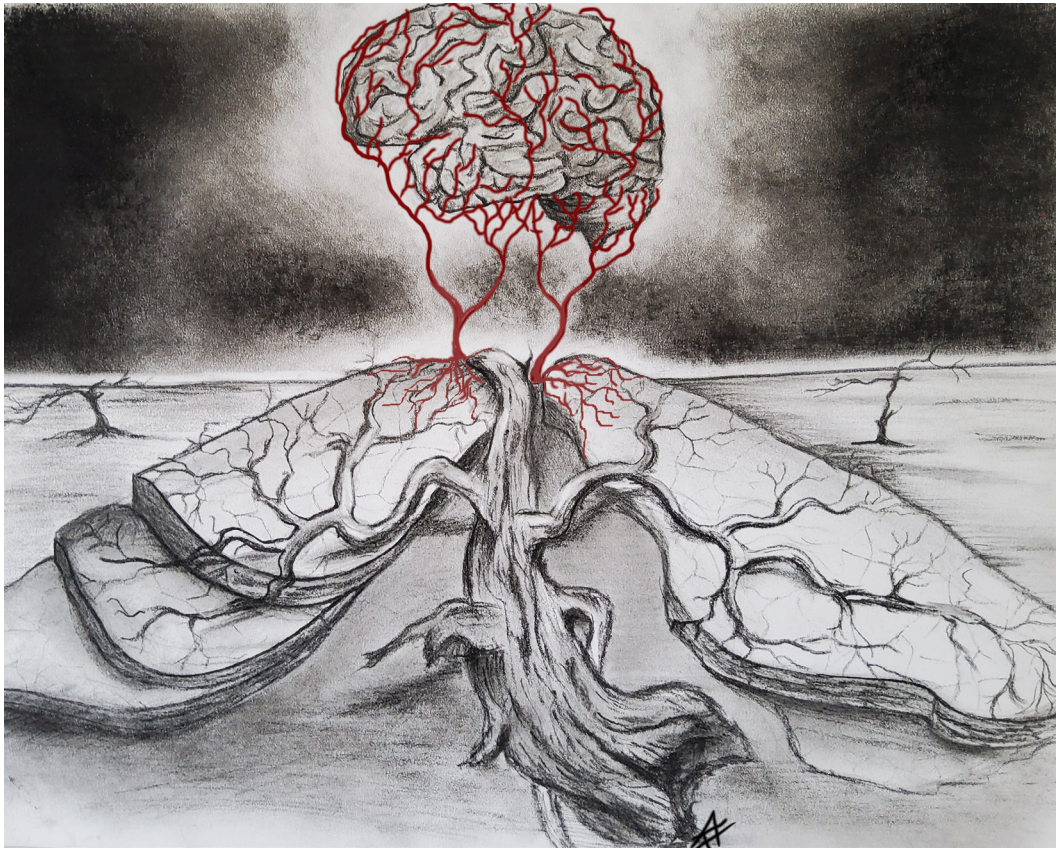


Biomarkers for CNS metastasis in Non-Small Cell Lung Cancer (NSCLC)



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Abstract

Non-small cell lung cancer (NSCLC) comprises more than 75% of lung cancer cases and is usually diagnosed in advanced stages. Lung cancer is the leading cause of cancer death among all solid tumours and the second most common malignancy globally. The prognosis of NSCLC patients with brain metastases (BM) is poor with a median overall survival of 4–11 weeks in untreated patients and 4–15 months in treated patients. Approximately 45% of patients with non-oncogenic driven NSCLC and 70% of patients with EML4-ALK rearrangements or EGFR mutations will be diagnosed with BM during the course of the disease. The incidence of BM appears to be increasing mostly owing to improvements in diagnostic imaging and in survival associated with more effective systemic therapies. The main purpose of this thesis is to identify clinical and molecular biomarkers for BM of NSCLC, as well as to explore the molecular diversity between CNS metastases and primary NSCLC.

Paper I was a single institution cohort study including brain metastasized lung cancer patients who received Whole Brain Radiotherapy (WBRT) at Karolinska University Hospital, Stockholm, Sweden. The aim of this trial was to find prognostic factors that can influence OS in lung cancer patients with BM treated with WBRT, in order to identify which patients will live long enough to experience the palliative benefit of WBRT, regarding disease control in the CNS. This study provided additional information on the selection of BM NSCLC patients who should receive WBRT by combining RPA and GRA prognostic indexes.

In **paper II** we analysed a total of 43 tissue samples from NSCLC patients for systematic mRNA expression; 13 primary tumours and 30 brain metastases. The material was obtained from 25 patients, of which 13 underwent surgery of the primary tumour. The paired samples were 26 (13 patients with both available lung and brain tissue samples). A unique gene downregulation pattern in brain metastases compared with primary tumours was observed. This finding may explain the lower intracranial efficacy of systemic therapy, especially immunotherapy, in the brain metastatic setting.

In **paper III** the validity of Lung-molGPA index in an ALK-positive lung cancer cohort with BM (n= 44) was explored, and a new prognostic scoring system, the ALK-BPI score, which can be easily applicable in clinical practice was proposed. PS, sex and BM at diagnosis, were used as prognostic variables in ALK-BPI.

The aim of **paper IV** - a retrospective cohort study consisting of 304 patients with surgically removed NSCLC- was to investigate whether high expression of NRF2 or TrxR1 in early stage NSCLC is predictive for relapse in CNS or other organs. High expression of NRF2 in cytokeratine positive cells in the whole tissue core compartment was correlated with higher risk for CNS relapse. This is to our knowledge the first study to report a predictive biomarker for CNS relapse in early stage NSCLC.

In summary, this thesis expands the knowledge regarding the molecular diversity between CNS metastases and primary NSCLC, and proposes new clinical and molecular biomarkers for BM of NSCLC.

LIST OF SCIENTIFIC PAPERS

- I. **Tsakonas, G.**; Hellman, F.; Gubanski, M.; Friesland, S.; Tendler, S.; Lewensohn, R.; Ekman, S.; de Petris, L. Prognostic factors affecting survival after whole brain radiotherapy in patients with brain metastasized lung cancer. *Acta oncologica* **2018**, *57*, 231-238, doi:10.1080/0284186X.2017.1386799.
- II. **Tsakonas, G.**; Lewensohn, R.; Botling, J.; Ortiz-Villalon, C.; Micke, P.; Friesland, S.; Nord, H.; Lindskog, M.; Sandelin, M.; Hydbring, P., et al. An immune gene expression signature distinguishes central nervous system metastases from primary tumours in non-small-cell lung cancer. *European journal of cancer* **2020**, *132*, 24-34, doi:10.1016/j.ejca.2020.03.014.
- III. **Tsakonas, G.**; Kamali, C.; De Petris, L.; Friesland, S.; Lewensohn, R.; Ekman, S. ALK-Brain Prognostic Index-Preliminary Study of a Prognostic Tool for Patients with ALK-Rearranged, Non-small Cell Lung Cancer and Brain Metastases. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12071804.
- IV. **Tsakonas, G.**; Martín-Bernabé, A; Moreno-Ruiz, P; Micke, P; Botling, J; De Petris, L; Ylipää, A; Mezheyeuski, A; Östman, A; Ekman, S. NRF2 expression in malignant cells of early stage NSCLC is predictive for relapse in the CNS. *Manuscript*

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LIST OF ABBREVIATIONS

ALK	Anaplastic Lymphoma Kinase
BM	Brain Metastasis
CI	Confidence Interval
CNS	Central Nervous System
CR	Complete Response
CT	Chemotherapy
DCR	Disease Control Rate
DS-GPA	Disease specific- Graded Prognostic Assessment
EC	Extracranial
EGFR	Epidermal Growth Factor
GPA	Graded Prognostic Assessment
HR	Hazard Ratio
IC	Intracranial
IHC	Immunohistochemistry
KPS	Karnofsky Performance Status
NRF2	Nuclear Factor Erythroid 2-related Factor
NSCLC	Non-small Cell lung cancer
ORR	Objective Response Rate
OS	Overall Survival
PFS	Progression Free Survival
PD(L)-1	Programmed Death Ligand 1
PR	Partial Response
PS	Performance Status
ROS-1	C-Ros Oncogene 1
RPA	Recursive Partitioning Analysis
RR	Response Rate
RT	Radiotherapy
RWD	Real World Data
SRS	Stereotactic Radiosurgery

TKIs	Tyrosine Kinase Inhibitors
TNM	Tumour Node Metastasis
TrxR1	Thioredoxin reductase 1
TTF	Time to Treatment Failure
WBRT	Whole Brain Radiotherapy

1 Background

1.1 Introduction-Epidemiology

Brain metastasis (BM) from solid tumours is more frequent than primary brain tumours. Dissemination in the central nervous system (CNS) occurs in up to 44% of patients with advanced non-small cell lung cancer (NSCLC), particularly in patients with adenocarcinoma [1]. Brain metastases are associated with a poor prognosis, with a median overall survival of 4–11 weeks in untreated patients and 4–15 months in treated patients [2, 3]. Especially patients with leptomeningeal carcinomatosis have a worse prognosis and therapeutic options are limited without any convincing palliative benefit in published studies; median overall survival not exceeding 2-3 months in treated patients and 4-6 weeks in untreated patients [4-6]. The blood–brain barrier is a physiologic obstruction to the delivery of systemic therapy to the central nervous system (CNS) and may contribute to treatment resistance.

The overall incidence of BM in advanced NSCLC increases to about 45% in the general patient population and up to around 70% of patients with EML4-ALK rearrangements or EGFR mutations will be diagnosed with BM during the course of the disease [1, 7-10]. The increasing incidence of BM is driven by the improvements in diagnostic imaging and survival due to more effective systemic therapies in the recent years [11].

1.2 Prognostic scores

Several prognostic scoring systems have been developed for patients with brain metastatic lung cancer. Recursive Partitioning Analysis (RPA) and Graded Prognostic Assessment (GPA) are the most widely used and validated scoring systems [12-14]. The RPA classification system was first published in 1997 and divided patients with brain metastases in three prognostic classes according to age, control of primary tumour, performance status measured according to Karnofsky Performance Scale (KPS) and the presence of extracranial disease. RPA was the first clinically important scoring system for NSCLC patients with BM [13, 14]. Thereafter, several prognostic scoring systems have been proposed for patients receiving WBRT or SRS (or both), adding parameters such as extracranial tumour control, interval between tumour diagnosis and WBRT start, number and volume of brain metastases, in relation to neurological outcomes and survival. [15-20]. The initial GPA-score was published in 2008 and has thereafter been

validated in other cohorts [21, 22]. The variables that proved to be significant in the GPA scoring system were Karnofsky performance status, age, presence of extracranial metastases, and number of brain metastases.

The disease-specific (DS) GPA, published in 2012, does not differ from the original publication regarding NSCLC patients with BM [23]. GPA was updated even regarding NSCLC patients with EGFR or ALK positive tumours. This lung-molGPA scoring system was published in 2016, based on 2,186 patients diagnosed with NSCLC and BM from 2006 to 2014 [24]. Sperduto et al. incorporated molecular characteristics (EGFR and/or ALK alterations) to previous significant variables, albeit lung-mol GPA has not yet been validated in large cohorts with only ALK- or EGFR-positive tumours [25]. A Chinese study published in 2017 failed to validate Lung-molGPA as a prognostic score [26].

1.3 Local Treatment of NSCLC with brain metastases

The majority of clinical trials evaluating the efficacy of surgery, whole brain radiotherapy (WBRT) and stereotactic radiosurgery (SRS) in metastatic brain tumours are not performed in lung cancer patients only. They consist of various primary tumours with different clinical courses and different metastatic burden. Therefore, it is difficult to draw safe conclusions for NSCLC, but on the other hand lung cancer patients are overrepresented in most of these trials as lung cancer has the highest frequency of brain metastases, as mentioned above [1, 27]. This is the reason for the wide application of the conclusions of such studies in lung cancer patients.

1.3.1 Surgery: in 2010, the first evidence-based compendium for the treatment of patients with brain metastases published a level 1 recommendation for surgical resection combined with radiation therapy to prolong life in relatively young patients with good functional status and a newly diagnosed solitary brain metastasis [28]. Prior to this formal guidance on the utility of surgery in patients with brain metastases, the benefits of this therapeutic option had been established in other studies [29, 30].

1.3.2 Whole-brain radiation therapy (WBRT): WBRT has been the standard-of-care for multiple NSCLC brain metastases for many years [31]. On the basis of a recursive partitioning analysis (RPA) of data from patients treated between 1979 and 1993 on previous RTOG protocols, even patients with brain metastasis who had the best prognosis had a median survival of only 7 months after WBRT alone[13]. The British Quartz trial, which included mostly RPA

class II and III patients, has shown no survival benefit, no difference in quality adjusted life years and no difference in corticosteroid use for patients with brain metastases from NSCLC who received WBRT, compared to dexamethasone and best supportive care only [32]. WBRT has not been shown to prolong survival in patients with leptomeningeal disease and its role in symptom palliation is controversial [33-35]. However, with improvements in systemic therapies for a variety of cancers, patient survival has now increased, even among those with metastatic disease [36]. In this context, WBRT alone is increasingly found to be inadequate in the long-term control of brain metastasis. In addition, with these improved outcomes many patients for whom control of brain disease is achieved with WBRT are surviving to experience the considerable neurocognitive sequelae and declines in quality-of-life that are associated with this treatment [37]. The classic neurocognitive toxicity associated with WBRT in adults is a moderate-to-severe dementia that occurs several months to years after treatment. De Angelis et al [38] observed a 2–5% incidence of severe dementia in populations of patients who had undergone WBRT (with or without surgical resection) for brain metastases, although these authors estimated that a markedly higher incidence of dementia would have been found if less severe cases of neurological decline were also included. An early neurocognitive decline, predominantly in verbal memory, occurring 1–4 months after WBRT has also been described [39].

1.3.3 WBRT and/or SRS: In order to avoid toxicity issues related to WBRT, SRS alone has been advocated in patients with better prognosis and a limited number of metastases [40, 41]. Two randomized studies [42, 43] have demonstrated that such patients (populations with one to three or four lesions) receiving SRS alone had a similar survival to patients who received WBRT and SRS. A series of meta-analyses of randomized controlled studies that investigated WBRT and SRS confirmed that WBRT did not enhance overall survival in patients with a limited number of brain metastases (up to four) [44]; however, reduced local and distant control of brain metastasis was observed after treatment with SRS alone compared with WBRT and SRS [44]. Indeed, as might be expected, patients treated with SRS alone do experience increased recurrences of metastasis elsewhere in the brain, but salvage with either repeat SRS or WBRT results in survival comparable to initial treatment with WBRT and SRS [44]. With respect to neurocognitive and performance outcomes, studies have demonstrated a considerable improvement in the preservation of neurocognitive function and performance status in patients treated with SRS alone compared with WBRT and SRS [43-46]. A randomized study by the European Organisation for Research and Treatment of Cancer (EORTC) further supports the

validity of using local therapy only (SRS or surgery) versus local therapy combined with WBRT in patients with one to three brain metastases, demonstrating no improvement in overall survival with the addition of WBRT [47]. Of note, patients in the WBRT arm of the EORTC trial scored markedly worse on health-related quality-of-life measures, both at early and late time points after treatment [42]. However, another randomized trial showed an overall survival benefit in univariate analysis for patients with one to three brain metastases who received WBRT and SRS compared to single WBRT, but this result was not validated in the multivariate analysis, and in a subgroup analysis the OS benefit was restricted to patients with single metastasis [48]. In the same study, Karnofsky Performance Status (KPS) at 6 months and local control were improved in the combined treatment arm, compared to single WBRT. A Cochrane meta-analysis regarding radiotherapy use for the treatment of multiple brain metastases has shown no difference in OS with combined SRS and WBRT versus SRS, and no OS difference with different fractionation and dosing of WBRT [49]. This meta-analysis showed better local control with combined WBRT and SRS and less side effects with single SRS. Pooled results from three randomized trials [43, 45, 50] (comprising a total of 364 patients) have revealed an apparent survival advantage in younger patients (< 50 years old) treated with SRS alone compared with WBRT and SRS [51]; the reason for this surprising result is not entirely clear at this time. At present, the use of SRS alone in patients with more than three metastatic lesions in the brain can be considered, although routine use of this approach in such instances will require validation in additional randomized controlled trials [52].

1.3.4 Adjuvant WBRT and/or SRS. In the adjuvant setting SRS or WBRT can increase local control rates with the latter having a greater impact on distant CNS recurrence-free survival, as shown in a meta-analysis [53]. This benefit in distant recurrence-free survival shown with WBRT is related to higher toxicity rates in the CNS, as mentioned above. A randomized trial by Patchell et al has shown better local and distant control in the CNS, as well as lower risk of death due to CNS recurrence for patients receiving adjuvant WBRT compared to observation only [54]. Up to date, studies of adjuvant RT in the CNS have been underpowered regarding OS and have not shown any such benefit.

1.4 Systemic Therapy of NSCLC with Brain Metastases

1.4.1 Chemotherapy: Early studies of traditional systemic chemotherapy agents showed clinical benefit in CNS metastasized NSCLC, especially in asymptomatic patients, often

enabling avoidance of local intervention such as surgery and radiation [55, 56]. Similarly, salvage therapy with cytotoxic chemotherapeutic agents owing to progression of disease after radiation therapy or surgical resection was also associated with clinical benefit, albeit modest [56]. Combination chemotherapy regimens, such as carboplatin with paclitaxel, cisplatin with vinorelbine, or carboplatin with etoposide, given in first line, have been shown to induce objectively measured responses of metastatic brain lesions in approximately 20–45% of patients with lung cancer, allowing delay of WBRT [57–59]. There is insufficient data to suggest the use of temozolomide monotherapy in the treatment of brain metastatic NSCLC, although some effect has been reported with dose-dense temozolomide in a small multicentre phase II trial [60], and also some activity has been observed against a variety of brain metastasized tumours [61].

In two phase III trials, examining concomitant topotecan or carboplatin with WBRT for brain metastasized lung cancer patients, no survival advantage was observed [62, 63]. A recent meta-analysis on the effect of concomitant temozolomide given with WBRT showed a modest increase in objective response rate (ORR), but failed to show any OS or PFS benefit [64].

1.4.2 Epidermal Growth Factor Receptor (EGFR) inhibitor therapy: *EGFR*-mutations are mainly detected in exons 18–21 of the *EGFR* gene encoding for the tyrosine kinase (TK) domain, and particularly the binding sites of EGFR tyrosine kinase inhibitors (TKIs) [65–67]. In more than 85% of cases, *EGFR* mutations are exon 19 deletions or the L858R point mutation in exon 21 [65, 67, 68]. These mutations are almost exclusively found in non-squamous NSCLCs, particularly papillary and lepidic adenocarcinoma [66]. In NSCLC, *EGFR* mutations are considered mutually exclusive from other molecular alterations, such as *K-Ras* and *HER2* mutations or *ALK* gene rearrangements, although coexistence of two or more driver mutations in the same patient can occasionally occur [69]. In NSCLC, *EGFR* mutations were reported in 5–10% of Whites, in 30% of White never-smokers, and more than 60% of Asian never-smokers. *EGFR* mutations are more common in women (20–62%) than men (1–19%) [66, 69].

First-generation EGFR TKIs erlotinib and gefitinib, second-generation afatinib and dacomitinib, and third-generation osimertinib, have been approved for the treatment of *EGFR*-mutated NSCLC in the first and later lines; for osimertinib in the second-line setting for tumours which develop resistance to first-line TKIs through the EGFR T790M mutation [70–81]. Orally administered gefitinib successfully controlled established intracerebral tumours derived from EGFR-expressing epidermoid cancers [82]. Gefitinib has also shown activity in NSCLC patients with brain metastases with unknown *EGFR* mutational status, with CNS disease control

rates ranging from 27% to 100% [83, 84]. However, clinical evidence for efficacy of these agents in tumours affecting the CNS comes mainly from subgroup analyses of randomized clinical trials, small prospective, retrospective observational studies and case series [82, 85-91] (Table 1). In general, first generation EGFR-targeting agents have a low capacity to penetrate into the cerebrospinal fluid (CSF), although erlotinib achieved relatively higher level of CNS penetration, which might in part explain the improved control of brain metastasis that has been observed with erlotinib treatment, even in patients previously treated with gefitinib (Table 1) [92]. Furthermore, the use of EGFR TKIs rather than chemotherapy as frontline therapy of *EGFR*-mutant lung cancer also reduced the cumulative incidence of progressive CNS metastasis (HR=0.56; 95% CI=0.34–0.94): 6-month, 12-month and 24-month cumulative risk of CNS metastasis was 1%, 6%, and 21%, respectively, in patients treated with EGFR-targeted agents compared with 7%, 19% and 32%, respectively, in those who received chemotherapy (P=0.026) [93]. In the FLAURA study, where osimertinib was compared to erlotinib or gefitinib in the first-line setting (N=556), patients with neurologically stable BM were allowed. In this trial, events of CNS progression were observed in 17 patients (6%) in the osimertinib group and 42 (15%) in the standard EGFR TKI group, irrespective of BM status at randomization [81]. A subgroup analysis of the FLAURA study done in 128 patients with BM at baseline showed that for osimertinib-treated patients, median CNS progression-free survival was not reached (95% CI, 16.5 months to not calculable) compared to 13.9 months (95% CI, 8.3 months to not calculable) with standard EGFR TKIs (HR=0.48; 95% CI, 0.26 to 0.86). CNS ORRs were 91% and 68%, respectively, in patients with one measurable CNS lesion (odds ratio=4.6; 95%CI, 0.9 to 34.9; P=.066) and 66% and 43%, respectively, in patients with measurable and/or non-measurable CNS lesions (odds ratio=2.5; 95% CI, 1.2 to 5.2; P = .011) treated with osimertinib or standard EGFR-TKIs [94].

Other prospective studies investigating the efficacy of first-generation EGFR TKIs in the treatment of brain metastasis have been mostly non-randomized single-arm phase II studies. One such study evaluated the role of erlotinib as a radiosensitizing agent in 40 patients with brain metastasis arising from lung cancer irrespective of the EGFR status of the tumour, and demonstrated the safety of this approach and a disease control rate of >80% (Table 1) [95]. Likewise, an *EGFR* mutation was detected in 53% of tumours with tissue available for testing, which is a high proportion when compared with the expected rate of *EGFR* mutations in Western populations of patients with lung cancer, suggesting that *EGFR* mutations can confer increased propensity for brain involvement. In the RTOG 0320 study, combination treatment

with WBRT, SRS and erlotinib/or temozolomide was compared to WBRT and SRS, showing that the combination treatment with TKI/or temozolomide and local RT had worse OS and higher toxicity [96]. The RTOG 0320 study was terminated prematurely due to poor accrual of patients and the patients included were not tested for *EGFR* mutation status, making it difficult to draw any clinically important conclusions.

In a study with NSCLC progressing after at least one line of chemotherapy and one line of EGFR TKI, patients received afatinib [97]. Afatinib appears to penetrate into the CNS with concentrations high enough to have clinical effect on CNS metastases and proved to be an effective treatment for heavily pre-treated patients with *EGFR*-mutated or EGFR TKI-sensitive NSCLC and CNS metastasis. Time to treatment failure (TTF) did not differ in patients with or without CNS metastases and over 70% of the patients with CNS metastases had either partial response (PR) or stable disease (SD), while 76% of the patients did not develop any new metastases. However, this study had many limitations, especially considering that the majority of patients had received WBRT before therapy with afatinib [97]. Afatinib has also shown a trend towards longer PFS compared to chemotherapy (8.2 vs 5.4 months) and higher DCR (92% vs 76%) in a combined subgroup analysis of the phase III LUX-LUNG 3 and LUX-LUNG 6 studies, including 81 patients who had CNS metastases (Table 1) [98].

The majority of patients with EGFR-mutant NSCLC, with initially good response to EGFR TKIs, acquires resistance to these drugs and develop CNS metastases, including leptomeningeal metastases (LM) [99, 100]. Several acquired resistance mechanisms, such as T790M secondary mutation [101, 102], *MET* amplification [103], and small cell transformation [104] have been reported. Pharmacokinetic failure due to insufficient penetration of standard-dose EGFR-TKIs is also regarded as a cause of CNS failure to EGFR-TKI [105]. High dose EGFR-TKIs has been considered as a reasonable therapeutic option for refractory CNS metastases after failure of standard-dose EGFR-TKIs, and several studies have suggested their effectiveness [87, 88, 106-110]. Indeed, high-dose erlotinib (1,500 mg weekly) was associated with a partial control of CNS metastases and stable disease in 67% and 11% of patients, respectively, in a retrospective series of nine individuals with EGFR-mutant lung cancer (Table 1); the median time to progression of CNS metastases was 2.7 months (range, 0.8–14.5 months), and the median OS was 12 months (range, 2.5 months to outcome not reached) [88]. Despite the clinical benefit shown with high dose TKI in the above mentioned studies, which are mostly case series or case reports with low evidence grade, there is an unmet need in finding new targeted treatments, especially for patients who develop acquired resistance mechanisms. Third-generation

irreversible EGFR-TKIs such as rociletinib, WZ-4002, and osimertinib have been recently developed and act as mutant-selective agents against both T790M (most common resistance mutation to first-generation TKI for exon 19 positive tumours) and the initial EGFR mutations but not against wild-type EGFR. [80, 111-113]. Data from the TIGER-X phase 1-2 study were promising regarding the effect of rociletinib in EGFR mutated lung adenocarcinoma patients, but further development of this drug was prematurely stopped [114]. Osimertinib is now considered a standard therapy for patients who develop resistance to first-/second-line EGFR TKIs through T790M-mutation in exon 20 [80, 115, 116] and is also indicated as first-line treatment in treatment-naïve advanced NSCLC patients with sensitizing *EGFR*-mutant tumours. These third- generation TKIs have shown activity against CNS metastases in the second-line TKI setting [117, 118]. Osimertinib achieves higher CSF/brain-to-blood ratio in mouse models than gefitinib, erlotinib or afatinib [119-121]. Data from TIGER-X study regarding the effect of rociletinib on CNS metastases were presented in ECCO 2015, showing ORR up to 45% and DCR up to 75% in the CNS [122]. In a pooled analysis of two phase II single-arm studies with osimertinib in T790M-positive NSCLC patients (AURA extension and AURA2, N=50), an intracranial ORR of 54% was observed, together with a 12% CR and a 92% DCR in the CNS [121]. In the phase III AURA3 study, osimertinib was compared to platinum/pemetrexed in EGFR-mutated tumours which had progressed on first-/second-generation EGFR TKIs (N= 419, n= 46 patients with measurable BM). In this trial, the CNS ORR to osimertinib was 70% vs 31% to chemotherapy. The median intracranial PFS was 11.7 months with osimertinib and 5.6 months with platinum/pemetrexed [HR=0.32; 95% CI, 0.15 to 0.69; P=0.004] [123]. A summary of clinical trials evaluating efficacy of EGFR TKIs in BM NSCLC is presented in Table 1.

Table 1. Trials evaluating efficacy of EGFR TKIs in NSCLC patients with BM.

Study	Treatment	Design	Number of patients	Prior therapy (number of patients)	ORR/DCR (%)	PFS (months)	OS (months)
Ceresoli et al [83]	Gefitinib	Prospective single arm phase 2	41	CT and WBRT in some patients	10/27	3	5
Wu et al [91]	Gefitinib	Prospective single arm phase 2	40	CT to all patients.	38/83	9	15

				WBRT or SRS: 26			
Welsh et al [95]	Erlotinib +WBRT	Prospective single arm phase 2	40	CT: 21 SRS: 4	83/86	8	11.8
Kim et al [90]	Erlotinib or gefitinib	Prospective	23	Treatment naive	70/83	7.1	18.8
Sperduto et al [96]	WBRT+SRS vs WBRT+SRS+Erlotinib(E) or temozolomide(T)	Prospective randomized phase 3	126 (381 planned)	NR	NR	WBRT/SRS: 8.1, WBRT/SRS/E: 4.8 WBRT/SRS/S/T: 4.6	WBRT/SRS: 13.4, WBRT/SRS/E: 6.1 WBRT/SRS/T: 6.3
Hotta et al [84]	Gefitinib	Retrospective	14	CT: All patients RT: 6	43/100	8.8	NR
Katayama et al [92]	Erlotinib	Retrospective	7	Gefitinib: All patients WBRT/SRS: 6 patients	43/86	NR	3
Grommes et al [88]	Erlotinib pulsatile weekly	Retrospective	9	WBRT/SRS: 3 Erlotinib daily: 4 CT: 3 No therapy: 3	67/78	2.7	12
Porta et al [89]	Erlotinib	Retrospective	17 EGFR mut, 52 EGFR unknown (control group)	WBRT: 55 No systemic therapy: 26	EGFR mut: 82/100 EGFR control: 0/78	EGFR mut: 11.7 EGFR control: 5.8	EGFR mut: 12.9 EGFR control: 3.1(whole control group, 16 early deaths)
Hoffknecht et al [97]	Afatinib compassionate use programme	Prospective	100, (61 EGFR actionable mut)	CT: All patients EGFR TKI: All patients	42/81 (in 31 out of 100 patients)	3.6 (TTF) 4.0 (TTF in EGFR mut)	9.8 (31% maturity)
Schuler et al [98]	Afatinib vs CT	Subgroup analysis of 2 prospective randomized trials	48 Afatinib, 33 CT	WBRT: 24 (13 in afatinib and 11 in CT group)	73/92	8.2 vs 5.4	22.4 vs 25
Varga et al [122]	Rociletinib	Prospective phase 2 trial	107 (T790M +)	CT: All patients TKI: All patients	45/75	NR	NR
Reungwetwattana et al [94]	Osimertinib (O) vs erlotinib/gefitinib(EG), first line	Subgroup analysis of randomized phase 3	128	Brain RT: 15 in (O), 16 in (EG) group	(O): 66/90, (EG): 43/84	(O): Not reached, (EG): 13.9	NR

		trial, FLAURA					
Goss et al [121]	Osimertinib	Pooled analysis of 2 phase II trials	50	Brain RT: 19, 1 st /2 nd generation TKI: All pat	54/92	Not reached	NR
Wu et al [123]	Osimertinib(O) vs platinum/pemetrexed(PP), second-line TKI	Subgroup analysis of randomized phase 3 trial, AURA3	46	Brain RT: 14 in (O) and 9 in (PP) arm, 1 st /2 nd generation TKI: All pat	(O): 70/90, (PP): 93/63	(O): 11.7 (PP): 5.6	NR

CT: Chemotherapy, PFS: Progression-Free Survival, OS: Overall Survival, TTF: Time to Treatment failure, WBRT: Whole Brain Radiotherapy, TKI; Tyrosine Kinase Inhibitor, SRS: Stereotactic Radiosurgery, ORR: Objective Response Rate, DCR: Disease Control Rate, RT: Radiotherapy, NR: Not Reported

1.4.3 ALK-targeted therapies: The EML4–ALK rearrangement is a genetic aberration that affects approximately 3–5% of all NSCLC, mainly younger, former light/no smoking history, and adenocarcinoma patients [124]. Of note, up to 30-70% of patients with ALK-positive lung cancer have been shown to develop brain metastasis [7, 8]. The ALK targeting agent crizotinib is the first approved agent for the treatment of ALK-positive NSCLC [125, 126]. The use of second-generation TKIs ceritinib, brigatinib and alectinib in the first- and second-line setting has shown similar efficacy both intracranially and extracranially, and seem to substantially reduce the cumulative risk of developing BM [8, 127-134].

Despite reports indicating poor penetration of crizotinib into the CSF [135], patients treated with ALK-targeted agents have frequently shown clinical response in the brain [125, 131], suggesting a potential role for these agents in the treatment of brain metastasis. Updated results from the PROFILE 1014 study showed a higher intracranial DCR at 12 and 24 weeks after treatment initiation, a higher ORR and longer PFS with crizotinib versus chemotherapy in the subgroup of patients with treated BM before study enrolment [136]. A retrospective analysis of the PROFILE 1015 and PROFILE 1007 trials with second-line crizotinib in ALK-rearranged lung adenocarcinoma studied the efficacy of this drug in BM disease [137]. Of 888 patients enrolled in these trials, 31% had asymptomatic BM. The DCRs at 12 weeks in the previously untreated or treated BM cases were 56 % (95 % confidence interval (CI) 46–66) and 62 % (95 % CI 54–70), respectively. ORRs in previously untreated or treated with radiotherapy were 18 % (95 % CI 5–40) and 33 % (95 % CI 13–59), respectively. The intracranial duration of response (DOR) in previously treated or untreated patients with an overall response was 26.4

(95 % CI 6.1–59.3) and not reached (95 % CI 6.0–59.9), respectively. Systemic PFS was not affected by the presence or absence of BM at baseline. Crizotinib is a substrate for the ATP-binding cassette (ABC) drug efflux transporters multidrug resistance protein 1 (also known as P-glycoprotein or ABC subfamily B member 1 [ABCG2]) and ABC subfamily G member 2 (ABCG2; also known as breast cancer resistance protein), which provides a potential reason for wide variability and overall poor accumulation of the drug in the brain [135]. In support of this theory, ABCB1^{–/–} and ABCG2^{–/–} mice had a 25–70-fold higher brain concentration following oral administration of crizotinib compared with wild-type mice [135]. Similar results were obtained when crizotinib was administered along with elacridar, an inhibitor of these efflux pumps [138]. These preclinical data, together with clinical evidence of intracranial efficacy of crizotinib [136, 139–142], indicates the potential utility of this drug for the treatment of brain metastasis of ALK-positive lung cancer.

In ASCEND-1 and -2 trials of ceritinib in ALK-rearranged lung adenocarcinoma, the intracranial DCR was 60.7% and 84.8% respectively, the overall intracranial RR was 35.7% and 39.4%, respectively, and median intracranial DOR 11.1 and 12.8 months, respectively, in patients who had previously received crizotinib [8, 130, 143]. ASCEND-3 trial has shown similar activity in ALK-rearranged lung adenocarcinoma patients with BM [144] (Table 2). These data were validated by the first-line study with ceritinib (ASCEND-4) which showed better IC ORR and PFS in ALK+ tumours in comparison with platinum-based chemotherapy (PFS by investigator assessment in BM patients: HR=0.58; 95% CI 0.36–0.92) [142]. Ceritinib has also shown activity in alectinib-pretreated patients with BM [145].

A phase 1/2 trial evaluated the efficacy of alectinib in patients with crizotinib-resistant ALK-rearranged NSCLC. In this trial, 11 out of 21 patients (52%) with CNS metastases at baseline achieved an ORR, and 5 out of 9 patients (56%) with measurable CNS lesions at baseline achieved a PR. Paired CSF and plasma samples of 5 patients showed measurable CSF concentrations of alectinib, and the drug showed promising activity even in patients with leptomeningeal dissemination [133]. In two phase 2 trials of alectinib in crizotinib-resistant ALK-rearranged lung adenocarcinoma the intracranial RR was 55.9% (34 patients with measurable CNS disease at baseline) [146] and 48% (69 patients with measurable CNS disease at baseline), respectively [134]. A pooled analysis of two of the above-mentioned single arm phase 2 studies with alectinib in crizotinib-resistant ALK-rearranged lung adenocarcinoma patients with CNS metastases included 50 patients (37%) with measurable CNS disease. 64% of these 50 patients had an OR (measured by independent review committee), DCR was 90%

and median DOR in CNS was 10.8 months. For all patients with CNS disease (both measurable and non-measurable CNS disease) the effect of alectinib was similar. ORR was 35.8% and 58.5% in patients with or without previous CNS radiotherapy, respectively [147]. In the ALUR trial, ALK-positive patients that had progressed on crizotinib and platinum CT were randomized to receive alectinib or CT (pemetrexed or docetaxel). Patients with measurable baseline CNS disease (alectinib, n=24; CT, n=16) treated with alectinib had a significantly higher CNS ORR (54.2%) versus those who received CT (0%; $P<0.001$) [148]. The multinational phase III study ALEX comparing alectinib versus crizotinib in treatment-naïve ALK-rearranged patients established alectinib as a first-line treatment choice. In this trial, the rate of CNS response to crizotinib in patients with measurable CNS lesions at baseline was 50% compared to 81% with alectinib, resulting in a cause-specific HR of 0.16 (95%CI 0.10-0.28). The median duration of CNS response was 5.5 months (95%CI: 2.1-17.3) with crizotinib and 17.3 months (95%CI: 14.8 to not estimable) with alectinib. Furthermore, the cause-specific HR for time to CNS progression was 0.18 for alectinib vs. crizotinib in patients with BM at baseline, and 0.14 for patients without baseline BM. These results indicate a reduction in the cumulative risk of developing BM in the alectinib arm [129]. This result is supported by the Japanese J-ALEX trial which had a similar design as the ALEX trial, where HR for CNS progression or death was 0.16 (95%CI: 0.02-1.28) for patients with BM at baseline and 0.41 (95%CI: 0.17-1.01) for patients without baseline BM for alectinib as compared to crizotinib [128].

Brigatinib has shown similar activity in ALK-rearranged CNS metastasized lung adenocarcinoma patients in a phase I/II trial, with 53% ORR (15 patients with measurable CNS lesions) and 87% intracranial DCR [149]. For all patients with an intracranial response (n = 19), median duration of intracranial response was 18.9 months. Brigatinib is now an established first-line treatment for ALK-positive NSCLC patients. In the randomized phase 3 trial ALTA-1L, brigatinib showed significantly superior PFS compared to crizotinib in treatment-naïve patients. This PFS benefit was consistent in subgroup analyses, including patients with BM. The estimated rate of 12-month survival without IC disease progression among patients with BM at baseline was 67% (95% CI, 47- 80) in the brigatinib group and 21% (95% CI, 6- 42) in the crizotinib group. The estimated rate of 12-month survival without IC disease progression in the intention-to-treat (ITT) population was 78% (95% CI, 68- 85) in the brigatinib group versus 61% (95% CI, 50- 71) in the crizotinib group. The rate of survival without IC disease progression among patients with baseline BM was higher in patients with brigatinib than with crizotinib (HR= 0.27; 95% CI, 0.13- 0.54) and the rate of survival without IC disease

progression among patients in the ITT population was higher in the brigatinib group than in the crizotinib group (HR= 0.42; 95% CI, 0.24- 0.70). An exploratory competing-risks analysis of IC disease progression, systemic progression, and death in the ITT population showed that the cause-specific hazard ratio for time to progression of IC disease was 0.30 (95% CI, 0.15- 0.60) [127]. The results of ALTA-1L trial are similar to ALEX and J-ALEX trials regarding the reduction of cumulative risk of developing BM during the course of the disease.

The novel second generation ALK-TKI ensartinib significantly prolonged progression-free survival over crizotinib with a favorable safety profile, in the global, phase 3, open-label, randomized eXalt 3 study. 290 patients with ALK- positive NSCLC who have received no prior ALK TKI and up to one prior chemotherapy regimen were randomized in this trial. The IC ORR was 64% with ensartinib versus 21% with crizotinib [150]. No robust clinical data for the efficacy of the third-generation TKI entrectinib in ALK-positive lung adenocarcinoma with BM are yet published. The third-generation ALK TKI lorlatinib has shown efficacy in BM with an ORR in target lesions reaching 60% in ALK- and ROS1-rearranged tumours [151]. Unpublished data from a phase 2 trial with lorlatinib in 57 heavily pretreated ALK-rearranged patients showed a promising IC ORR of 54% with IC DOR 12.4 months [152]. Lorlatinib showed an impressive IC ORR of 82% and IC CR of 71%, in patients with measurable BM in the global phase 3 randomized CROWN study. In this trial, lorlatinib was compared to crizotinib in treatment naïve patients with ALK-positive NSCLC, and showed a statistically significant and clinically meaningful improvement in PFS vs crizotinib. HR for time to intracranial progress was 0.07 (95% CI: 0.03-0.17) in favor of lorlatinib compared to crizotinib [153]. Results from larger ALK-positive BM cohorts regarding the IC efficacy of third-generation TKIs are eagerly expected. A summary of clinical trials evaluating efficacy of ALK TKIs in NSCLC with BM is presented in Table 2.

Table 2. Trials evaluating efficacy of ALK TKIs in NSCLC patients with BM.

Study	Treatment	Design	Number of patients	Prior therapy (number of patients)	Intracranial ORR/DCR(%)	Intracranial Response Duration	PFS (months)
Solomon et al [136]	Crizotinib vs CT	Subgroup analysis of a randomized phase 3 trial	79 (39 vs 40)	No prior systemic therapy	77/85(12m), 56(24m) vs 28/45(12m), 25(24m)	15.7m vs 12.5m	9 vs 4
Costa et al [137]	Crizotinib (RT treated BM vs untreated BM)	Subgroup analysis of 2 randomized 3 trials	275 (166 vs 109)	CNS RT: 166 CT: All patients	33/62 vs 18/56	Not Reached vs 26.4w	6 vs 5.9

Kim et al [130]	Ceritinib	Subgroup analysis of a phase 1 trial	98	CT:All pat ALKi: all pat CNS RT: 68	36/61 (in pat with measurable BM)	11.1m (in pat with measurable BM)	6.7
Kim et al [130]	Ceritinib	Subgroup analysis of a phase 1 trial	26	CT: 23 CNS RT: 15	63/63 (pat with measurable BM)	8.2m (in pat with measurable BM)	18.4
Crino et al [8, 143]	Ceritinib	Subgroup analysis of a phase 2 trial	100	CT: all pat Crizotinib: all pat	39.4/84.8 (pat with measurable BM)	12.8m	5.4
Felip et al [144]	Ceritinib	Subgroup analysis of a phase 2 trial	50	CT: All pat CNS RT: 27	58/86	9.1m	10.8
Soria et al [142]	Ceritinib vs CT	Subgroup analysis of a randomized phase 3 trial	121 (54 vs 52)	CNS RT: 37 vs 40	46.3/88.9 vs 21.2/91.3	16.6m vs not estimable	13.5m vs 6.7m
Gadgeel et al [133]	Alectinib	Subgroup analysis of phase 1/2 trial	21	CT: 1 CNS RT: 4 Crizotinib: all pat	52/80	NR	NR
Gadgeel et al [147]	Alectinib	Pooled analysis of 2 phase 2 trials	136	CT: 109 CNS RT: 95 Crizotinib: all pat	42.6/85.3(all pat) 64/90(pat with measurable BM)	11.1m(all pat) 10.8m(pat with measurable BM)	8.3
Novelo et al [148]	Alectinib vs CT	Subgroup analysis of a randomized phase 3 trial	40 (24 vs 16)	CT: all pat Crizotinib: all pat	54.2/79.2 vs 0/31.3	Not reached vs 0	NR
Peters et al [129]	Alectinib vs crizotinib	Subgroup analysis of a randomized phase 3 trial	43 (21 vs 22)	No prior systemic therapy	81/NR vs 50/NR	17.3m vs 5.5m	NR
Rosell et al [149]	Brigatinib	Subgroup analysis of a phase 1/2 trial	15 (pat with measurable BM)	CNS RT: 6 Systemic therapy:NR	53/87	18.9m (in 19 pat with CNS response)	NR
Camidge et al [127]	Brigatinib vs crizotinib	Subgroup analysis of a randomized phase 3 trial	90(43 vs 47)	No prior systemic therapy	79/NR vs 23/NR	NR	NR
Selvaggi et al [150]	Ensartinib vs crizotinib	Subgroup analysis of a randomized phase 3 trial	104 (48 vs 56)	No prior ALKi	64/NR vs 21/NR	NR	NR

Solomon et al [151]	Lorlatinib	Subgroup analysis of a phase 1/2 trial	39(ALK+, ROS1+)	ALKi: Most of the pat CT: NR CNS RT: NR	60/NR (in 20 target lesions)	NR	NR
Solomon et al [153]	Lorlatinib vs crizotinib	Subgroup analysis of a randomized phase 3 trial	78 (38 vs 40), 30 pat with measurable BM (17 vs 13)	No prior systemic therapy	66/NR vs 20/NR, 82/NR vs 23/NR (pat with measurable BM)	NE vs 9.4m, NE vs 10.2m (pat with measurable BM)	NR

CT: Chemotherapy, PFS: Progression-Free Survival, CNS RT: Central Nervous System Radiotherapy, ALKi: ALK Inhibitor, ORR: Objective Response Rate, DCR: Disease Control Rate, pat: patients, BM: Brain metastases, NR: Not Reported, NE: Not Evaluable.

1.4.4 Immunotherapy in NSCLC with BM: Currently, anti PD-(L)1 inhibitors such as nivolumab, pembrolizumab and atezolizumab, are approved for pre-treated advanced NSCLC [29,30,60,61]. Single agent pembrolizumab, with or without platinum-based chemotherapy, is currently approved as first-line treatment for advanced NSCLC patients without EGFR/ALK molecular aberrations [62-65]. The quadruplet carboplatin/paclitaxel/bevacizumab/atezolizumab is another first-line approved regimen, with efficacy data also in the EGFR-/ALK-positive population [66].

Patients with NSCLC and BM were underrepresented in most of these registration studies, where only asymptomatic cases were included. In the second-line setting, few patients with asymptomatic BM were included in the trials with PD-1 inhibitors nivolumab and pembrolizumab versus docetaxel. The small subgroup analyses evaluating these patients did not show any clinical benefit with immune checkpoint inhibitors (ICIs) compared to single agent docetaxel. The OAK trial, investigating the PD-L1 inhibitor atezolizumab, showed a longer OS compared to docetaxel in the subgroup of patients (85 out of 850) with asymptomatic BM (HR=0.54; 95% CI: 0.31-0.94) [154-157] (Table 2). In the KEYNOTE-024 trial, the subgroup of patients without BM at baseline (172 out of 305) had a better PFS with pembrolizumab versus chemotherapy (HR=0.50; 95% CI: 0.36–0.68) [158]. In the KEYNOTE-407 trial comparing chemotherapy with or without pembrolizumab in the first-line setting of advanced squamous cell NSCLC, no subgroup analysis was presented for patients with asymptomatic BM at baseline (44 out of 559) [159]. A retrospective exploratory subgroup analysis of the KEYNOTE-189 trial (platinum/pemetrexed +/- pembrolizumab in the first-line

setting for advanced non-squamous NSCLC), presented at the 2019 annual American Association of Cancer Research (AACR) meeting, evaluated survival outcomes among patients with BM at baseline. 108 of 616 patients (17.5%) had treated and/or stable BM at baseline and received platinum/pemetrexed or platinum/pemetrexed plus pembrolizumab. The chemotherapy/ICI combination showed a longer OS in patients with BM (7.5 vs 19.2 months, HR 0.41, 95% CI, 0.24-0.67) and without BM (12.1 vs 22.4 months, HR 0.59, 95% CI, 0.46-0.75). HR for PFS were also in favour of the chemotherapy/ICI combination treatment with an improvement of median PFS from 4.7 to 6.9 months (HR 0.42, 95% CI, 0.27-0.67) in patients with BM and an improvement from 4.9 to 9.2 months (HR 0.48, 95% CI, 0.39-0.59) without BM[160]. The combination treatment was well tolerated in patients with BM and no new safety signals were identified. The OS and PFS benefit of the chemotherapy/ICI combination observed in patients with BM was similar to that seen in the overall population, adding evidence to the role of this regimen in the first-line setting in this subgroup.

Interim analysis of the non-randomised phase 2 study by Goldberg *et al.* evaluating pembrolizumab in small (5-20mm), asymptomatic BM was presented at ASCO 2018. Thirty-nine patients (34 with PD-L1 positive and 5 with PD-L1 negative/unevaluable tumours) were treated with pembrolizumab. Pembrolizumab was given as first, second or later line of treatment in 30.8%, 35.9% and 33.3% of patients, respectively. In PD-L1 positive patients, IC ORR, IC PFS (among patients with response or SD in the BM) and median OS were 29.4% (95% CI 15.1-47.5), 10.7 months (95% CI 6.6- not reached), and 8.9 months (95% CI 6.6-29.7 months), respectively. There were no IC responses in patients with PD-L1 negative or un-evaluable tumours [67].

A recently published retrospective analysis evaluated the efficacy of ICI in 1025 patients with NSCLC treated in 6 European centers [68]. Two hundred and fifty-five patients (14.3% were symptomatic) had BM at initiation of ICI therapy. With a median follow up of 15.8 months, there was no difference in ORR (20.6% vs 22.7%; $P = .484$) for patients with or without BM. IC ORR was 27.3%, and IC DCR was 60.3%. Median PFS was 1.7 months in patients with BM, compared to 2.1 months in patients without ($P = .0009$). The median OS was 8.6 vs. 11.4 months for patients with and without brain metastases, respectively ($P = .035$). These results suggest that ICI is active in patients with BM and these patients should not be excluded from future clinical trials.

A case series with 5 patients with NSCLC and asymptomatic BM treated with nivolumab showed 1 CR, 1 PR and 1 SD. IC and systemic responses were largely concordant and time to

response ranged between 5 and 9 weeks [161]. A retrospective study with 241 NSCLC patients with BM, treated with PD-(L)1 inhibitors, showed an IC ORR of 27.3% in the subgroup with active BM (new or growing, 40% of all BM patients). The IC ORR was higher in PD-L1+ tumours (35.7%) vs. PD-L1- tumours (11.1%). Overall (IC+EC) ORR was 22.7% in patients without BM versus 20.6% in patients with BM ($p=0.484$). Median (95% CI) PFS and OS in patients without vs. patients with BM were 2.1 (1.9-2.5) vs. 1.7 (1.5-2.1) months and 13 (9-16) vs. 9 (7-13) months, respectively. The use of corticosteroids, PS ≥ 2 and more than 2 metastatic sites were associated with worse PFS and OS in the multivariate analyses [162].

Promising results with ICI in BM have been demonstrated using real world data. The nivolumab expanded access programme (EAP) in Italy enrolled 372 squamous NSCLC patients, of which 38 had asymptomatic, controlled BM. The DCR in BM patients was 47.3% (1 CR, 6 PR and 11 SD), compared to 47% in the overall population. Median PFS and OS in BM patients were 5.5 and 6.5 months, respectively [163]. The Italian nivolumab EAP for non-squamous NSCLC showed similar results. In 409 patients out of 1588 (26%) with asymptomatic BM, nivolumab achieved a DCR of 40% (3 CR, 65 PR, 96 SD) and a median OS of 8.1 months (95% CI 6.2-10.1), without increased toxicity [164]. An observational multicentre French study in 43 NSCLC patients with BM showed an IC ORR of 9% [95% CI: 3–23%]) and EC ORR of 11% [95% CI: 4–26%] with nivolumab. The IC DCR was 51% (95% CI: 37–66%) and did not differ significantly from the EC DCR (47% [95% CI: 31–62%]) ($p=0.43$). Patients were heavily pre-treated and median overall and IC PFS were 2.8 (95% CI: 1.8–5.3) and 3.9 (95% CI: 2.8–11.1) months, respectively. Median OS was 7.5 (95% CI: 5.6–not reached) months. [165]. A retrospective series evaluating the impact of nivolumab in NSCLC patients with BM, presented at WCLC 2018, showed a similar IC ORR of 10.4%, with an overall ORR of 20.8%, and a median duration of response of 11.5 months. IC PFS and OS were 8 and 9 months, respectively [166]. In recent work by Kim et al., IC and EC responses to ICI in lung cancer with BM were compared. In 11 patients that achieved a PR or SD in the primary lung tumour, eight showed a progression in BM. Interestingly, PD-1+ tumour infiltrating lymphocytes (TILs) were significantly decreased in BM compared to primary lesions, possibly contributing to a lack of responses in BM [167] (Table 3).

Table 3. Immunotherapy effect in NSCLC with BM

Study	Treatment	Design	Number of BM patients	Prior Therapy (number of patients)	ORR (%) [IC if not otherwise specified]	PFS (months)	OS (months)
Rittmeyer et al [157]	Atezolizumab vs docetaxel	Randomized phase 3 trial, subgroup analysis	85/850	Platinum based CT, 640 pat 1 line, 210 pat 2 lines	NR	NR	20.1 vs 11.9
Goldberg et al [168]	Pembrolizumab	Phase 2 trial	18	72% systemic therapy, 56% local CNS therapy	IC: 33, EC:33	NR	7.7
Dudnik et al [161]	Nivolumab	Case series	5	3 CNS RT	40	NR	NR
Bidoli et al [163]	Nivolumab	Nivolumab expanded access programme in Italy, squamous NSCLC	38/372	At least one line of CT	18	5.5	6.5
Crino et al [164]	Nivolumab	Nivolumab expanded access programme in Italy, non-squamous NSCLC	409/1588	At least one line of CT in all pat	17	NR	8.1
Hendriks et al [162]	PD(L)-1 inhibitors	Retrospective trial	241/945	At least one line systemic therapy	27.3 in 96 pat with	1.7	9

				[range 1-12],68% CNS RT	active BM (see text)		
Gauvain et al [165]	Nivolumab	Retrospective observational trial	43	79% local CNS therapy, at least one line systemic therapy	IC: 9, EC:11	IC PFS: 3.9, Global PFS: 2.8	NR
Geier et al [166]	Nivolumab	Retrospective trial	77	At least one line systemic therapy [range 1-6], CNS RT in 48 pat	10.4	8	9
Goldberg et al [67]	Pembrolizumab	Phase 2 trial	39	30.8% initial 35.9% 1 prior systemic therapy 33.3% 2+ lines therapy	29.4%	10.7	8.9
Garassino et al[64]	Pembrolizumab	Retrospective analysis	108/616	Treatment naive	NR	6.9	19.2
Kim et al [167]	Nivolumab or pembrolizumab	Retrospective analysis	18	All pat metastasectomy and CT,72% CNS RT	11	NR	NR

CT: Chemotherapy, PFS: Progression-Free Survival, IC: Intracranial, EC: Extracranial, CNS RT: Central Nervous System Radiotherapy, SRS: ORR: Objective Response Rate, OS: Overall Survival, PD(L)-1: Programmed Death Ligand 1, pat: patients, BM: Brain metastases, NR: Not Reported.

1.4.5 Other clinically actionable mutations: New data has emerged the past few years for the treatment of NSCLC (lung adenocarcinoma specifically) with novel TKIs. ROS1 fusion oncogene is detected in 1-2% of lung adenocarcinoma patients [169] and drugs like crizotinib

and lorlatinib have shown clinical activity in small patient populations [170-172], but no published data exist regarding activity in the CNS, except for the above mentioned unpublished data for lorlatinib [151]. The combination of the BRAF inhibitor dabrafenib with the MEK inhibitor trametinib has shown clinical activity in pre-treated BRAF mutated patients (V600E, 1-2% of lung adenocarcinoma patients) in a phase 2 trial [173], but no data from clinical trials exist regarding CNS activity. Our research group has recently published a case report on a patient with primary CNS metastatic BRAF mutated lung adenocarcinoma with complete intracranial response to BRAF/MEK inhibition [174]. Since dabrafenib has shown activity in brain metastatic melanoma [175], it is highly probable that the same activity is going to be observed in lung adenocarcinoma patients with CNS disease, but more data are needed from clinical trials where BRAF mutated BM lung cancer patients are included. Other therapies targeting HER-2 mutations or amplification, MET exon 14 skipping mutation or amplification, RET fusions, NTRK fusions and other oncogenic drivers are in early clinical trials with variable results [176]. Results regarding activity of these compounds in BM of NSCLC are eagerly expected.

1.5 Cellular antioxidant response and carcinogenesis

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is a vital component of cellular antioxidant response and its activation in cancer cells promotes cancer progression and metastasis [177-179]. In pancreatic cancer cells, NRF2 supports cell proliferation and metabolism through the regulation of cap-dependent and cap-independent mRNA translation [180]. A key study by DeNicola et al showed that NRF2 promoted K-RAS^{G12D}-initiated pancreatic and lung tumorigenesis as well as proliferation in cancer cell lines and human pancreatic cancer tissue. Furthermore, KRAS^{G12D}, BRAF^{V619E}, and c-MYC^{ERT2} oncogenic signaling was related to increased mRNA and protein levels of Nrf2 and its target genes [181]. The role of NRF2 expression is most probably mediated through the promotion of expression of certain genes that are vital for cell proliferation and metabolism, such as *NOTCH1*, *NPNT*, *BMPRIA*, *IFG1*, *ITGB2*, *PDGFC*, *VEGFC*, and *JAG1* [180, 182, 183]. A recently published trial from Taiwan showed that cytoplasmic NRF2 expression in early stage NSCLC was correlated with worse prognosis and worse response to cisplatin-based chemotherapy after relapse [184]. In another trial done in early stage NSCLC patients, cytoplasmic NRF2 expression, as well as expression of its stabilizing protein DJ1, were independent prognostic factors for poorer OS [185]. Thioredoxin reductase 1 (TrxR1) is the

cytosolic isoenzyme of the three different TrxRs found in human cells. Its role in cancer biology is less clear since it may protect normal cells from carcinogenesis, but may also promote cancer progression [186].

2. Aims of the thesis

The main purpose of this thesis is to identify clinical and molecular biomarkers for BM of NSCLC, as well as to explore the molecular diversity between CNS metastases and primary NSCLC. The aims of each paper are described below.

Paper I. Paper I aimed to find prognostic factors that can influence OS in lung cancer patients with BM treated with WBRT, in order to identify which patients will live long enough to experience the palliative benefit of WBRT, regarding disease control in the CNS.

Paper II. In this paper, gene expression analysis was carried out from formalin-fixed, paraffin-embedded (FFPE) tissue sections of primary lung tumours and matched brain metastases. The aim was to identify potential patterns of gene downregulation or upregulation in the BM setting that could potentially explain the dismal prognosis of these patients, function as a diagnostic tool and potentially aid the development of new treatment strategies.

Paper III. In this study the validity of Lung-molGPA index in an ALK-positive lung cancer cohort with BM was explored, and a new prognostic scoring system which can be easily applicable in clinical practice was proposed.

Paper IV. The aim of the fourth study was to investigate whether high expression of NRF2 or TrxR1 in early stage NSCLC is predictive for relapse in CNS or other organs. We focused mainly on the risk for CNS relapse due to the dismal prognosis of this patient category.

3. Patients, materials and methods

3.1 Patient cohorts

Paper I was a single institution cohort study including 280 brain metastasized lung cancer patients who received WBRT at Karolinska University Hospital from the first of January 2010 until the first of January 2015. Information about Recursive Partitioning Analysis (RPA) and Graded Prognostic Assessment (GPA) scores, demographics, histopathological results and received oncological therapy were collected.

For paper II, patients with lung cancer with surgically removed BM were identified from two cohorts. The first cohort consisted of 725 patients with early-stage NSCLC, who received surgical treatment from 1/1/1995 to 30/12/2010 at Akademiska University Hospital in Uppsala, Sweden. The second cohort was the same which was used in paper I, consisting of 280 patients who had received WBRT during the course of their disease. We analysed a total of 43 tissue samples for systematic mRNA expression; 13 primary tumours and 30 brain metastases. The material was obtained from 25 patients, of which 13 underwent surgery of the primary tumour. The paired samples were 26 (13 patients with both available lung and brain tissue samples).

Paper III was a retrospective cohort study, where initially 106 patients with advanced ALK-positive NSCLC who were treated between January 2009 to November 2019 at Karolinska University Hospital in Stockholm, Sweden were reviewed. 54 patients were diagnosed with BM and 44 patients received ALK-TKI as first-line therapy after BM diagnosis. The latter patient group was included in our analyses. We collected demographic data, information about given oncological therapy, histopathology and physicians' evaluations of performance status before receiving ALK TKI for brain metastatic disease.

Paper IV was a retrospective cohort study, consisting of 304 patients with surgically removed NSCLC. Patients received surgical treatment from 1/1/2006 to 30/12/2010 at Akademiska University Hospital in Uppsala, Sweden. Demographic data were collected retrospectively. We collected information about physician's evaluation of performance status (PS) at the time of diagnosis, age at diagnosis, histology, relapse site, surgical stage of disease, smoking status and gender. The tissue micro array (TMA) cohort was based on diagnostic tissue from NSCLC patients operated at the Akademiska University Hospital in Uppsala between 1995 and 2010,

from the same cohort that was used in paper II. 258 patients were included in the final analysis due to missing data regarding tumour relapse.

All studies have received ethical approval by the institutional review board/ethical committee in Stockholm (registration numbers: 2016/944-31/1, 2019-03658) and the ethical committee in Uppsala (registration numbers: 2006/325, 2012/532). Additional approvals by Stockholm Biobank (Bbk01605) and Uppsala Biobank (BbA-827-2018-058) were received.

3.2 RPA, GPA and Lung-molGPA classifications

We divided patients in RPA classes and GPA groups according to age, control status of primary tumour, KPS, number of BM and presence of extracranial disease. All patients in paper III were ALK positive and this was used in Lung-molGPA calculation. In paper I, brain MRI with contrast was performed in 85% of the patients. The rest of the patients (15%) had only CT-verified metastases, but all of these patients had >8 BM, therefore not influencing GPA scoring. In the same paper patients were divided into three GPA groups; group 0 (0-1 points), group 1 (1.5-2.5 points) and group 2 (3-4 points), where group 0 was the worst prognostic group and group 2 the best prognostic group. In paper 2, brain MRI was available for all patients and GPA classification was done as described above for paper I. Brain MRI with contrast was performed in 68% of the patients in paper III and the rest of the patients (32%) had CT-verified metastases. However, all of the patients with CT-verified metastases had >4 BM, therefore not influencing the GPA scoring. Patients were divided into four Lung-molGPA groups; group 1 (0-1.0 points), group 2 (1.5-2.0 points) group 3 (2.5-3.0) and group 4 (3.5-4.0 points). In the same paper, patients were divided into four GPA (DS-GPA) groups; group 1 (0-1.0 points), group 2 (1.5-2.5 points), group 3 (3.0) and group 4 (3.5-4.0 points). Group 1 was the worst prognostic group and group 4 the best prognostic group in both Lung-molGPA and DS-GPA (Table 4). Presence of extracranial disease was confirmed with CT scan of the thorax and abdomen in all patients in papers I-III. CT thorax and abdomen was performed ≤ 4 weeks from the time of BM diagnosis.

Table 4. RPA, GPA (=DS-GPA) and Lung-mol GPA classifications

<i>RPA class</i>	Class 1	Class 2	Class 3
	Age < 65 years, KPS ≥ 70, primary tumour under control, no extracranial metastases	All other patients	KPS<70
GPA			
Prognostic factor	Scoring criteria		
	0	0,5	1,0
Age, y	>60	50-59	<50
KPS	<70	70-80	90-100
Number of BM	>3	2-3	1
ECM	yes		No
Lung-molGPA			
Prognostic factor	Scoring criteria		
	0	0,5	1,0
Age, y	≥70	<70	
KPS	<70	70-80	90-100
Number of BM	>4	1-4	
ECM	Yes		No
Gene Status	EGFR neg/unk and ALK neg/unk		EGFR or ALK pos

ECM: Extracranial Metastases, KPS: Karnofsky Performance Status, EGFR: Epidermal Growth Factor receptor, ALK: Anaplastic lymphoma kinase, neg: negative, unk: unknown

3.3 RWD analysis and outcomes of patients

A major concern in modern oncology is the external validity of the results from large randomized control trials (RCTs). The patients that are included in these RCTs are generally younger, with better PS and without comorbidities, something which rarely is the case in the clinical praxis [187, 188]. The purpose with real world data analysis (RWD) is to collect data and analyse the patients outcome in a real world setting, thus helping regulatory decision making and guiding clinicians in their daily praxis [189].

The papers in this thesis represent RWD analyses. This is the reason for the different GPA scoring between papers I-III, due to the low number of patients in the best prognostic group. In the clinical praxis, brain metastatic NSCLC patients with lower GPA score and RPA class 2 or 3 are more common. The internal validity of RWD can be questioned, since information and

selection bias are unfortunately quite common. Missing data for some analyses was a problem in paper I, III and IV. The selection of some patients depending on the patient cohort used for the analyses is another limitation. Patients with worse prognosis are included in the WBRT cohort compared to patients with early stage disease in the surgical cohort. Despite these limitations which are known and cannot be avoided, RWD analyses play an important role in modern oncology and can assist clinicians regarding decision making.

3.4 Immunohistochemistry/TMAs

Immunohistochemistry (IHC) was conducted in tissue microarrays (TMA) from surgically removed lung tumours. Haematoxylin-eosin stained slides were reviewed by two pathologists and tumour areas to be included in the TMA were marked. No major discrepancies regarding IHC scoring between the two pathologists were observed. The TMA was constructed using a manual tissue arrayer (MTA-1, Beecher Instruments, Sun Prairie, CA) [190, 191]. All tumours were included in duplicates (2 x 1 mm tissue cores). 4 micrometer sections were cut from the TMA blocks, mounted on adhesive slides and baked in 60°C for 45 min. TMA blocks were successfully constructed for all 304 patients, but we could include only 258 in our analysis due to missing clinical data regarding tumour relapse. NRF2 and TrxR1 expression was measured in cancer cells in the whole tissue, in the tumour area and in surrounding stroma separately. Cancer cells were defined as epithelial cells that expressed cytokeratin (CK+). The epithelial density was measured with CK expression in the whole tissue, tumour and stroma.

3.5 Gene expression analyses

Formalin-fixed, paraffin-embedded (FFPE) tissue sections of primary tumours and matched brain metastases were used for total RNA isolation, using RNeasy FFPE kit (Qiagen, Hilden, Germany). Tissue sections of 4 X 4 µm were used. RNA quantity and quality were assessed using RNA Screen Tapes on a 2200 TapeStation system (Agilent, Santa Clara, CA, USA) through the documentation of RNA integrity number curves.

An nCounter FLEX™ Analysis System (nanoString, Seattle, WA, USA) using the nCounter® PanCancer Immune Profiling gene expression panel (nanoString Technologies Inc.) was used for the measurement of systematic mRNA expression. This panel covers 770 human mRNAs

associated to tumour- and immunity-related pathways. A minimum input of 150 ng of total RNA was used for each sample. Fluorescently colour-coded reporter probes and biotin-labelled capture probes were hybridized to the mRNA on a thermal cycler overnight and automatically processed and loaded to the nanoString provided sample cartridge in the nCounter Prep Station.

3.6 Statistical analysis

For the characterization of the cohorts in all papers descriptive statistical methods were used. The normally distributed variables were presented as mean \pm standard deviation (SD), while non-normally distributed variables are presented as median (interquartile range, IQR). The normality of all continuous variables was assessed by skewness and kurtosis. Protein expressions in paper IV were also tested with receiver operating characteristics (ROC) curves in order to find an optimal cut-off value. Kaplan–Meier curves were plotted to determine OS from different time points until death depending on the trial design of each paper. Patients who were alive at the time of data collection were treated as censored observations during the analysis. Curves were compared with the log-rank test. Subgroup OS analysis were performed when needed. In paper I subgroup OS analysis was stratified by RPA class.

Predictors of OS were identified by Cox regression analyses. Univariate Cox regression analyses were undertaken in all papers including different variables depending on the respective trial design. The results from the univariate analysis with a significant *p* value, as well as variables considered clinically significant guided the selection of variables for the multivariate Cox regression analysis. In paper I, non-significant variables were removed from the model by stepwise backward selection and the multivariate analysis was done separately for RPA class and GPA class, due to overlapping.

In paper II, mRNA expression normalization, hierarchical clustering, scatter plots, fold-changes and statistical ranking of differentially expressed genes, along with FDR corrected *p*-values, were performed using the nanoString nSolver analysis software (nanoString Technologies Inc.). mRNA raw expression counts were normalized to the top 100 genes and not to housekeeping genes. Hierarchical clustering was performed using Euclidean distance with average linkage. Normalized expression values of the top twelve differentially expressed mRNAs were extracted from all samples. Scatter plots and curves displaying ROCs along with values for area under curve (AUC) were acquired using Graphpad Prism 8. All

differentially expressed genes were subjected to KEGG term analysis, including calculation of Benjamini-Hochberg corrected p-values, through the miRWalk 3.0 software. Visualization of KEGG gene interaction networks was performed through the Net NetworkAnalyst 3.0 software. Specifically, the node network was visualized through the layout settings “Force Atlas” and “Reduce Overlap”.

For the calculation of our new prognostic score in paper III, *ALK-Brain Prognostic Index (BPI)*, maximum score of 1.0 was given to factors with larger effect estimates, while maximum score of 0.5 to smaller effect estimates. C-statistics were implemented and a bootstrap validation with 1000 samples was performed. The mean C-statistic over the bootstrap samples was used as a measure of model performance, which also was statistically compared between the prognostic scores. The Kaplan–Meier approach with the log-rank test was then used to compare overall survival between the groups within the prognostic scores.

The primary outcome was relative risk for relapse in CNS or other organ after surgery in paper IV and it was calculated by binary logistic regression. Odds ratios (OR) with 95% confidence intervals (CI) were calculated in univariate and multivariate regression analyses. Univariate logistic regression analyses were undertaken with NRF2 expression in cytokeratin positive (CK+) cells in different TMA compartments, TrxR1 expression in CK+ cells in different TMA compartments, and other clinically important parameters as independent variables. The variables that were found to be significant in predicting the risk for relapse, as well as variables that were deemed to be clinically significant predictors for the risk of relapse were included in the multivariate regression models. Fisher’s exact test was also implemented in order to test the correlation between tumour relapse and protein expression (high versus low).

All statistical analyzes were done with SPSS versions 23 and 25 (IBM Corp, Armonk, NY, USA), as well as R version 3.61.

4. Results and discussion

4.1 Prognostic factors affecting survival after whole brain radiotherapy in patients with brain metastasized lung cancer (paper I)

In this single cohort RWD analysis 280 patients who received WBRT for brain metastatic lung cancer were included. 54.7% of patients died in the first 3 months after the start of WBRT. Pairwise log rank testing showed statistically significant differences in OS with $p < .0001$ between RPA classes 1–3 and 2–3 as well as GPA groups 0–1. The best prognostic GPA group 2 had a very low number of patients, something which influenced the results of all statistical analyses. A statistically significant longer OS was observed for patients < 70 years ($p < .0001$). Subgroup OS analyses were performed for RPA classes taking into account GPA groups. The most interesting finding was in RPA class 2 subgroup ($n = 165$), where a statistically significant OS difference was observed between GPA group 0 (79 patients) and GPA group 1 (78 patients) with $p = .004$.

Univariate survival analysis showed that age (both continuous and dichotomous variable with 70 years as cutoff), open surgery in CNS before salvage WBRT, RPA class and GPA class group had a statistically significant impact on OS. In the multivariate analysis including RPA class, we found age and RPA class to be independent prognostic factors. In the multivariate analysis including GPA class, the independent prognostic factors were CNS surgery before salvage WBRT, age, symptomatic CNS disease and GPA group. A multivariate Cox regression analysis was also performed in RPA subgroup 2 ($n = 165$), which showed that age, CNS surgery before salvage WBRT and GPA group (1 vs. 0) were independent prognostic factors for OS.

In order to further explore the heterogeneity in RPA class 2 subgroup, we performed a Kaplan–Meier OS analysis taking into account the different age and GPA groups. We divided the combined groups as described below; Group A (‘intermediate group’): age ≤ 70 and GPA group 0 [41/165], group B (‘good group’): age ≤ 70 and GPA group 1 or 2 [66/ 165], group C (‘intermediate group’): age > 70 and GPA group 1 or 2 [20/165] and group D (‘bad group’): age > 70 and GPA group 0 [38/165]. The pairwise log rank test showed a statistically significant difference in OS between groups A and D, as well as B and D. The median OS in days was 193 [95% CI: 147–239] (B group), 118 [95% CI: 55–181] (A group), 90 [95% CI: 18–162] (C group) and 89 [95% CI: 51–127] (D group).

In the majority of the existing prognostic scoring systems for BM patients receiving cranial irradiation there are considerable drawbacks, mainly caused by the limited number of patients studied and the heterogeneity regarding the kind of radiotherapy used (WBRT or SRS) [15-20]. In this real life cohort study, RPA and disease specific GPA were used, since they are the most practical, most validated and most used scoring systems for patients with BM from solid tumours [22]. The median OS found in RPA classes and GPA groups in our cohort are similar to those reported in the literature [13, 21, 22, 192]. The exception was GPA group 2 (GPA score 3-4) where we found a slightly worse OS than group 1, due to the low number of patients in group 2 ($n=13$).

RPA classification has received criticism since it depends mostly on PS, and due to heterogeneity regarding prognosis mostly in RPA class 2 [15, 193]. In our cohort median OS for RPA class 3 was 41 days (95% CI 35-49 days), something which supports the omission of WBRT in this patient group. Median OS was 324 days (95% CI: 197-451 days) for RPA class 1 patients, to whom WBRT should be given if clinically indicated. It is of major clinical interest to find a way to identify which patients should receive WBRT in the heterogeneous RPA 2 class. WBRT has a palliative effect regarding CNS disease control, but not any proven OS benefit. Therefore, it is important to identify which patients will live long enough to overcome the early toxicity related to WBRT and experience its palliative benefit [49, 194]. We found that age over 70 years and GPA score can help choosing the right patient to receive WBRT in the heterogeneous RPA 2 subgroup. In the intermediate prognostic groups of RPA 2 patients, with either age ≤ 70 years and GPA < 1.5 points, or age > 70 and GPA ≥ 1.5 points, age seems to be a stronger prognostic factor for OS compared to GPA. This real life cohort study showed that WBRT should be considered in a larger proportion of BM NSCLC patients compared to QUARTZ trial results, where all patients older than 65 years (not RPA class 1) do not seem to derive any benefit from WBRT [32].

The major limitations of this paper are its retrospective nature and the small number of patients in some subgroup analyses, making it prone to selection bias. A large number of patients died early after the completion of WBRT, something which constitutes the selection of “healthier” patients less likely. The majority of the subgroup analyses done in this paper had a relatively large number of patients, and in the cases where the number was low, no conclusion can be drawn. The recommendations from our study results regarding the correct use of WBRT is applicable only in patients who benefit most from WBRT.

4.2 An immune gene expression signature distinguishes central nervous system metastases from primary tumours in non-small-cell lung cancer (paper II)

In paper II, we identified patients with lung cancer with surgically removed BM from two cohorts. The first cohort consisted of 725 patients with surgically removed NSCLC and the second of 280 patients who had received whole brain radiotherapy during the course of their disease (i.e. the cohort used in paper I). From these 1005 patients who were screened, a total of 43 tissue samples was available for systematic mRNA expression; 13 primary tumours and 30 brain metastases. This material was obtained from 25 patients, of which 13 underwent surgery of the primary tumour. The paired samples were 26 (13 patients with both available lung and brain tissue samples).

Tissue samples were divided into two groups, primary and CNS metastasis, and subjected to mRNA array analysis using the nanoString Pan-Cancer Immune Oncology panel followed by ranking of transcripts with differential expression between the groups. mRNA expression between these groups was significantly altered (FDR corrected p-value <0.05) in 208 mRNAs (of totally 770). All differentially expressed transcripts displayed decreased expression in brain metastases compared to primary tumours, albeit with a large variation in relative fold-changes (1.43- 96.70). Brain metastases clustered in a distinct manner compared to primary tumours regarding the fraction of differentially expressed genes. This pattern was substantiated when only including genes with a corrected p-value of <0.01 , or when specifically selecting the top twelve most differentially expressed genes. Additionally, the mRNA expression of all genes from patients which had available both a primary tumour and a brain metastasis sample was analyzed. The mRNA expression was largely decreased in the brain metastatic lesion compared with the primary tumour, irrespective of investigated patient.

Top differentially expressed genes, CCL19 and CCL21, were substantially dampened in the brain metastatic lesion. Of the top twelve most differentially expressed mRNAs, seven belonged to the chemokine (CCL19, CCL21, CXCL9, CCL14, CCL18) or cytokine receptor families (IL2RB, IL21R). The additional five most differentially expressed transcripts were CD48, GZMA, ITGA4, RUNX3 and TPSAB1. The diagnostic performance of our top differentially expressed mRNAs was investigated by computing their ROC scores. The best performing individual transcripts were CCL19 and CCL21 with an AUC score of 0.9795 and 0.9667, respectively. All top twelve differentially expressed transcripts scored an AUC >0.90 , with four of them scoring an AUC >0.95 (CCL19, CCL21, IL2RB and TPSAB1).

Finally, an *in silico* pathway association of all transcripts being differentially expressed in brain metastatic lesions was performed by computing their KEGG term associations. 35 KEGG terms had a highly significant p-value ($p < 0.0001$, Benjamini-Hochberg corrected). Of these KEGG terms, “Cytokine-cytokine receptor interaction” achieved the highest number (56) of differentially expressed transcripts, of which seven were found among the top twelve differentially expressed transcripts. This KEGG-term analysis on differentially expressed genes revealed a concomitant enrichment of multiple KEGG-terms associated with the immune system.

In paper II, a unique gene downregulation pattern in the brain metastatic setting compared to primary lung cancer was identified. These genes either belong to the cytokine and chemokine receptor families or are otherwise involved in immune response and immune cell activation. A statistically significant downregulation of several genes encoding for checkpoint proteins, including PDCD1 (encoding the PD-1 protein), CTLA4, LAG3 and ICOS was observed [195]. The role of CCL19 and CCL21, the most differentially expressed genes in our study, is to recruit Tregs, CD4 T-helper and activated T-cells, monocyte-derived dendritic cells and B-cells to the tumour microenvironment through interaction with the chemokine receptor CCR7, another differentially expressed gene in our study.

An excellent diagnostic performance was observed for 12 genes in the ROC curves which were implemented for the evaluation of the differential gene expression. This 12-gene signature clearly separated brain metastases from primary tumours and several clinical applications of this signature are possible in the future. These applications can be related to the diagnosis of brain metastatic NSCLC from solid biopsies, monitoring of patients during treatment (especially with immunotherapy) or the use of this diagnostic signature in liquid biopsies (plasma or cerebrospinal fluid) when tumour material is not available.

One limitation of our study is that sequencing was not performed in order to identify relevant mutations and in a second step correlate mutations with gene expression. Furthermore, no specific analyses of the tumor microenvironment were performed. Nevertheless, this study is one of only two studies in literature up-to-date which have shown an immunosuppressive environment in the brain metastatic setting, when matched gene expression analyses from primary lung cancer and brain metastasis were compared [196]. The results from paper II may explain the low intracranial response rates and the limited efficacy of ICIs for patients with brain metastatic NSCLC. This may open up for new treatment strategies of brain metastases

in NSCLC where modulation of chemokine and cytokine signaling can be used to create an “immune-rich” tumour microenvironment optimized for a response to ICIs.

4.3 ALK-Brain Prognostic Index - a Prognostic Tool for Patients with ALK-Rearranged Non-Small Cell Lung Cancer and Brain Metastases (paper III)

Paper III aimed to explore the validity of the Lung-molGPA index, as well as the possibility to develop a new prognostic scoring system in ALK-positive lung cancer patients with BM. For this reason, 44 patients who received ALK-TKI as first-line therapy after BM diagnosis were included in the study, after a screening of 106 ALK positive patients.

Univariate analysis of clinically important variables showed that primary brain metastatic disease (HR=0.17; 95% CI: 0.05-0.58), PS (HR= 0.32; 95% CI: 0.13-0.79) and sex (HR=0.40; 95% CI: 0.17-0.95) were prognostic factors for OS. All these variables proved to be statistically significant independent prognostic factors for OS in the multivariate analysis and were included in our prognostic score, ALK-Brain Prognostic Index (ALK-BPI). In the design of ALK-BPI, maximum score of 1.0 was given to variables with larger effect estimates, such as primary brain metastatic disease (HR= 0.14; 95% CI: 0.04-0.48), and maximum score of 0.5 to smaller effect estimates, such as PS ≤ 3 (HR=0.29; 95% CI: 0.11-0.79) and female sex (HR=0.27; 95% CI: 0.11-0.67). Patients were divided into two prognostic groups; the good prognosis group (1.5-2.5 points) with a median OS of 65.7 months (95% CI: 47.3-84.1) and the poor prognostic group (0-1.0 points) with a median OS of 22.7 months (95% CI: 14.8-30.5).

The different prognostic scores included in our analyses, ALK-BPI, Lung-molGPA and DS-GPA were compared initially with log-rank test which was 0.0068, 0.006 and 0.0619, respectively. The univariate analysis of the different prognostic scores showed that DS-GPA prognostic groups were not significant for OS, not all Lung-molGPA groups differed significantly, while there was a clear difference between the two prognostic groups in the ALK-BPI. A bootstrap analysis with 1000 bootstrap samples was further undertaken. The mean C-statistics of the different prognostic scores were 0.6354, 0.6772 and 0.6796 for the ALK-BPI, Lung-molGPA and DS-GPA, respectively, and when compared to each other with the p-value no significant difference was observed.

Prognostic scores for lung cancer patients with BM, such as RPA, GPA and DS-GPA, were initially calculated using patient data from clinical study cohorts and their external validity can therefore be questioned [12, 13, 23]. In Lung-molGPA, the number of ALK-positive patients included in the statistical analysis was not given and EGFR-mutation positive patients were calculated together with ALK-positive patients. Another major limitation of the Lung-molGPA is that available treatment options for ALK-positive lung cancer patients were limited and considered obsolete in daily clinical praxis nowadays (patients diagnosed between 2006 and 2014) [24]. The number of BM and presence of extracranial metastases were not significant prognostic factors for ALK-positive patients in our study, a result which is in concordance with the results from a Chinese study, which also failed to validate the Lung-molGPA score [26].

The major limitations of our study are its retrospective nature and the relatively small number of patients included. On the other hand, this is the first prognostic score calculated in a homogeneous ALK cohort receiving ALK-TKI as first-line treatment for brain metastatic disease. The RWD analysis of our study strengthens the external validity of ALK-BPI. The results of our study are closer to what observed in daily clinical practice, where the number of BM and presence of extracranial disease do not seem to influence OS when patients are treated with second generation ALK TKIs. The results of the Kaplan-Meier analyses discriminate the ALK-BPI groups in a better way compared to the other prognostic scores, and the results from the bootstrap analysis with 1000 samples failed to show any statistically significant difference between the different prognostic scores. DS-GPA performed worst in all analyses without any statistical significance in log-rank test or univariate analysis.

The three variables (primary brain metastatic disease, sex and PS) which are used in ALK-BPI, are easily calculated in the clinical setting without the need of radiological evaluations. This important feature, together with the fact that ALK-BPI has only two prognostic groups with a clear difference in OS between them, renders it an attractive prognostic tool.

4.4 NRF2 expression in early stage NSCLC is predictive for relapse in CNS (paper IV)

In **paper IV**, multiplex IHC staining was implemented in surgical biopsies from early stage NSCLC patients. NRF2 and TrxR1 expression was measured in cancer cells in the whole tissue, in the tumour area and in surrounding stroma separately. Cancer cells were defined as epithelial cells that expressed cytokeratin (CK+). The epithelial density was measured with CK expression in the whole tissue, tumour and stroma. The primary outcome was relative risk

for relapse in CNS or other organ after surgery and it was calculated by binary logistic regression.

We had information about NRF2 and TrxR1 expression in the different TMA compartments for 304 patients. 258 were included in the final analysis due to missing data regarding tumour relapse. 56.6% of these 258 patients did not experience a relapse, while the most common relapse site was the thoracic cavity (22.9%). 16 patients had relapse in the CNS (6.2%). The expression of TrxR1 in CK+ cells in the different TMA compartments failed to show any statistically significant risk for relapse, relapse only in the CNS or relapse in other sites. The same analyses done for NRF2 expression showed a significantly higher risk for relapse in the CNS, which was observed in CK+ cells in the whole biopsy compartment, with an OR of 7.36 (95% CI: 1.64- 33.06) and a chi-square p-value of 0.003. The OR for relapse in the CNS was 3.08 (95% CI: 0.97- 9.81) for the NRF2 expression in CK+ cells in the tumour compartment, and 3.05 (95% CI: 0.96- 9.72) in the stroma compartment. Chi-square p-values for the abovementioned compartments were 0.068 and 0.069, respectively. NRF2 expression was not correlated with higher risk for relapse in general in the different CK+ TMA compartments.

Despite not being significant in the univariate analysis, age, histology and stage were deemed to be clinically significant variables and were included in the multivariate analysis together with the CK+/NRF2+ expression in the whole tissue. The CK+/NRF2+ expression in the whole biopsy was an independent positive predictive factor for CNS relapse with an OR of 8.00 (95% CI: 1.70- 37.60), whereas none of the other variables were statistically significant.

The median value of NRF2 expression was initially tested as a cut-off to define high versus low expression, in order to have an unbiased first analysis. The distribution of NRF2 expression in the CK+ cells in the whole biopsy compartment was not normally distributed with a skewness of 4.6 and kurtosis of 28.7. The ROC analysis failed to show another optimal cut-off value and the median IHC expression of NRF2 was decided to be the most optimal cut-off for our cohort.

NRF2 expression in CK+ cells in the surgical biopsy was an independent prognostic factor with 8-fold higher odds regarding risk for relapse in the CNS in our cohort of early stage lung cancer patients. To our knowledge, this is the first study to report a predictive biomarker for CNS relapse in early stage lung cancer. NRF2 expression seems to play a more important role in cancer progression and metastasis. This role is most probably mediated through the

promotion of expression of certain genes that are vital for cell proliferation and metabolism [180, 182, 183]. A recently published trial from Taiwan showed that cytoplasmic NRF2 expression in early stage NSCLC was correlated with worse prognosis and worse response to cisplatin-based chemotherapy after relapse [184]. In another trial done in early stage NSCLC patients, cytoplasmic NRF2 expression, as well as expression of its stabilizing protein DJ1, were independent prognostic factors for poorer OS [185]. In our study we have not evaluated the cytoplasmic expression of NRF2, albeit NRF2 expression in the different tumour compartments in different cell types did not influence OS.

The major limitation of our study is its retrospective nature making it prone to selection bias. A certain risk of information bias regarding missing follow-up data about tumour relapse also exists. Information bias regarding IHC scoring cannot be avoided, albeit considered limited in our cohort due to the methodology used (see methods). Overfitting of data was avoided in the first step of our analyses by choosing median IHC expression value as a cut-off in order to define high versus low protein expression. Further statistical analyses with ROC curves and descriptive analysis of the IHC staining distribution did not reveal any other optimal cut-off value. This methodology renders the protein expression analysis as unbiased. The size of our cohort was large enough in order to perform the planned statistical analyses, even though the number of patients with CNS relapse was relatively small, something which is expected in a cohort of early stage NSCLC patients. The real-world nature of our study strengthens the external validity of our results. Nonetheless, these results should be validated in an external cohort before they can be implicated clinically.

5. Conclusions

The results from **paper I** suggest that the selection of brain metastatic lung cancer patients who are candidates for WBRT could be done in two steps. RPA classification is a useful and practical prognostic tool for making a first step assessment. WBRT should be omitted for RPA class 3 patients without further evaluation, whereas RPA class 1 patients should receive WBRT, if clinically indicated. The second step assessment is solely for RPA class 2 patients, where we suggest that patients with age ≤ 70 years and GPA ≥ 1.5 points should be treated as RPA 1 patients. WBRT should be omitted in RPA 2 patients with age > 70 . In RPA 2 patients with age ≤ 70 years and GPA < 1.5 points WBRT could be a reasonable option.

In **paper II**, a unique gene downregulation pattern in brain metastatic NSCLC samples compared to primary tumours was identified, especially regarding genes related to immune response and immune cell activation. This finding may explain the lower intracranial efficacy of systemic therapy, especially immunotherapy, in NSCLC patients with BM, and opens up for new treatment strategies. Furthermore, a 12-gene signature with excellent diagnostic performance for brain metastatic NSCLC was identified.

Paper III proposes the new ALK-BPI score as a prognostic tool that can easily be applied for ALK-positive lung cancer patients with BM in daily clinical practice, and that has at least the same, if not better, prognostic value compared with the Lung-molGPA. The ALK-BPI score should be validated in a larger cohort consisting of brain metastatic ALK-positive patients receiving ALK-TKI in the first-line setting.

Paper IV is the first study to report a predictive biomarker for CNS relapse in early stage NSCLC and the first trial to report the correlation between NRF2 expression and risk for CNS relapse. The external validation of these results is needed. The results of our trial could alter the follow-up strategy of early stage NSCLC patients and eventually improve OS for NRF2 positive cases.

6. Future perspectives

Despite the major advances in the diagnosis, treatment and radiologic imaging of NSCLC patients during the past decades, there is still an unmet need in identifying clinical and molecular biomarkers that will aid clinicians in choosing tailored treatments in an individualized setting. This need is even more profound in NSCLC patients with BM, due to their dismal prognosis and the low IC efficacy of most existing systemic and local therapies [194].

The results of **paper I** are important regarding the selection of NSCLC patients with BM who should receive WBRT. There is an abundance of prognostic scores for this category of patients, some of which have major limitations and have not been widely applicated in the clinical praxis, perhaps with the exception of RPA and GPA [14, 22]. In our RWD analysis, age over 70 years was an independent prognostic factor for OS after WBRT. This result is concordant with our institution's experience regarding brain irradiation of elderly patients and also with published studies in elderly patients with primary CNS tumours [197, 198]. Age over 70 years and GPA score can help choosing the right patient to receive WBRT in the heterogeneous RPA 2 subgroup. This finding can help clinical decision making in this frail patient category, albeit new prognostic biomarkers are needed. The combination of molecular biomarkers with clinical prognostic scores could be the optimal strategy in future trials for NSCLC patients with BM.

Paper II was focused on identifying potential patterns of gene downregulation or upregulation in the BM setting of NSCLC patients, something that could explain the dismal prognosis of this patient category, function as a diagnostic tool and constitute new treatment strategies. The effect of systemic therapies in the CNS is limited for NSCLC patients and new treatments as well as predictive biomarkers are eagerly needed [57-59, 158]. The registration trials evaluating single ICI for advanced NSCLC have included small numbers of asymptomatic BM patients and data from such subgroup analyses, as well as from retrospective series have shown limited activity of ICI in BM NSCLC [161, 163-166, 168]. The unique gene downregulation pattern in BM compared to primary tumors which was observed in our study may explain the low intracranial response rates and the limited efficacy of immune checkpoint inhibitors (ICI) for patients with BM NSCLC. Multiple genes encoding for immune checkpoint proteins displayed substantially dampened expression in the BM samples compared to primary tumour. This may open up for

new treatment strategies of BMs in NSCLC where modulation of chemokine and cytokine signaling can be used to create an “immune-rich” tumour microenvironment optimized for a response to ICIs. In order to achieve higher response rates in the CNS, new treatment strategies are needed. Combination treatments with two different ICIs, or ICI and other drugs influencing the immune microenvironment/immune cells in the CNS, seem to be a reasonable approach [199, 200]. Furthermore, the 12-gene signature for brain metastatic NSCLC which was identified in our study had an excellent diagnostic performance and will be subject for further validation studies in other patient cohorts.

The ALK-BPI proposed in **paper III** is a new prognostic tool that can easily be applied for ALK-positive lung cancer patients with brain metastases in the daily clinical practice, and that has at least the same prognostic value as Lung-molGPA. The older prognostic scores for NSCLC patients with BM, described in paper I, are considered obsolete regarding ALK-positive disease. Lung-molGPA included EGFR and ALK-positive patients in the same group, something which is a major disadvantage since these two molecular entities differ substantially. In addition to that, patients included in Lung-molGPA received oncologic treatment before the introduction of new generation TKIs, which are considered standard of care today. These observations underline the need for an updated prognostic score for ALK-positive NSCLC patients with BM. The validation of the ALK-BPI score in a larger cohort, consisting solely of brain metastatic ALK-positive patients receiving ALK-TKI in the first-line setting, is an ongoing project at our clinic. Clinically meaningful prognostic scores, such as ALK-BPI, can contribute to the identification of patients with poor prognosis and eventually lead to the development of new treatment strategies.

There is a lack of predictive and prognostic biomarkers in early stage NSCLC. **Paper IV** is the first study to report a correlation between NRF2 expression and risk for CNS relapse in early stage NSCLC. The early detection of CNS relapse may prove to be of vital importance for this patient category. The detection of CNS relapse when patients are asymptomatic, something which is translated clinically into a much better PS, have less tumour burden in the CNS and perhaps no extracranial disease, will most probably lead to an OS benefit. This assumption is derived from the evidence regarding the prognosis of these patients, where surgery for single metastasis, better PS, lower number of CNS metastases and the absence of extracranial disease is associated with a longer OS [23, 194]. The follow-up strategy for patients that have higher risk for CNS relapse after surgery should include brain MRIs in regular intervals. Nonetheless, these results should be validated in an external cohort before they can be implicated clinically.

In conclusion, there is an unmet need regarding biomarker research for NSCLC patients with BM. All these patients should be included in randomized clinical trials, something which is unfortunately not a routine nowadays. The combination of clinical and preclinical biomarker research expertise is of vital importance in order to narrow the gap between these two research fields, and in order to accelerate the identification of clinically important biomarkers.

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